

Induction of secretory immunity with bioadhesive poly (D,L-lactide-co-glycolide) microparticles containing *Streptococcus sobrinus* glucosyltransferase

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The effect of mucosal delivery of *Streptococcus sobrinus* glucosyltransferase (GTF) in bioadhesive poly (D,L-lactide-co-glycolide) (PLGA) microparticles on induction of salivary IgA and serum IgG antibody responses was measured in Sprague-Dawley rats. Preparations of GTF/PLGA/gelatin microparticles, or PLGA/gelatin microparticles or GTF in alum, were administered four times at weekly intervals by intranasal or intragastric routes. Two subcutaneous injections of GTF in PLGA/gelatin microparticles or in alum were given to separate groups of rats. Significant elevations in salivary IgA antibody levels to *S. sobrinus* GTF were observed only in the groups immunized intranasally 28 days after immunizations were begun. Five of six rats given the GTF microparticles intranasally had positive salivary IgA antibody responses to GTF, and the mean salivary IgA antibody level of this group was 30-fold higher than any other mucosally or systemically immunized group. Salivary IgA responses in the GTF-microparticle group remained significantly higher than all other mucosally immunized groups for at least 10 weeks after the primary immunization. All rats in this group demonstrated aspects of anamnesis following a more limited secondary course of intranasal administration. Intranasal administration of GTF in microparticles also induced a serum IgG response to GTF in some rats. After secondary intranasal GTF microparticle administration, several rats had sustained serum IgG antibody levels that were within the range of sera from rats subcutaneously injected with GTF in microparticles or in alum. Thus intranasal delivery of GTF-containing bioadhesive microparticles induced the highest and longest lasting salivary immune response of any mucosal or systemic route or vehicle tested and could be expected to be a useful method for induction of mucosal immunity.

Key words: PLGA; microparticles;
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Glucosyltransferases contribute to the molecular pathogenesis of mutans streptococci, chiefly because of their ability to synthesize extracellular glucan from sucrose (5). Strategies for immune intervention in the processes leading to dental caries, therefore, have included the development of immune responses to glucosyltransferase (GTF) (14). Glucan synthesis, which is the consequence of the catalytic action of GTF on sucrose, is an important component of the colonization/accumulation potential of mutans streptococci, and, thus the expression of the virulence of these organisms (5). Glucosyltransferases (GTF) from mutans streptococci can induce immune responses that inhibit GTF catalytic activity (18), protect rodents from experimental dental caries (13, 19), and interfere with reaccumulation of indigenous mutans streptococci in humans (15, 16). These levels of experimental protection from mutans streptococcal colonization and disease can be achieved by topical (16), oral (13, 15) or intragastric (13) application of GTF, although levels of demonstrable IgA antibody induced tend to be low and relatively short-lived.

A continuing issue in mucosal vaccine therapy is the problem of delivering the vaccine to the site of antibody induction in an efficient and undegraded state. One approach to this problem is to incorporate antigen into microcapsules for immunization. Microspheres and microcapsules made of poly(D,L-lactide-co-glycolide) (PLGA) have been used as local delivery systems (1, 2, 7) because of their ability to control the rate of release, evade pre-existent antibody clearance mechanisms and degrade slowly without eliciting an inflammatory response to the polymer. Particularization of antigen also optimizes association with M cells overlaying inductive regions of the secretory immune system (3, 11). However, the use of microcapsules as delivery vehicles for protein antigens has been somewhat problematic since most formulations require the use of organic solvents, for example methylene chloride, which can denature the antigen and render much of it biologically inactive. Limited bioadhesion and inappropriate particle size for uptake by M cells have also limited the usefulness of this approach. However, a modification in PLGA microparticle manufacture has addressed the problem of antigen degradation by incorporating antigen

into microparticles in an aqueous phase (6). Increasing the bioadhesion of PLGA microparticles has also been shown to enhance particle uptake (20). These antigen-loaded microparticles, may, therefore, have the potential to induce a long lasting mucosal immune response. Thus, the present study was designed to explore the ability of microparticles containing *S. sobrinus* glucosyltransferase as antigen, and 1% gelatin as bioadhesive, to induce mucosal immunity by intranasal, intragastric or subcutaneous routes of administration.

Material and methods

Glucosyltransferases

GTF from *Streptococcus sobrinus* strain 6715 was obtained as previously described (18). After bacterial growth in glucose-containing defined medium, enzymes were isolated from culture medium by affinity chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) with 3 M guanidine HCl as the eluting solvent. This GTF-rich pool was then subjected to FPLC liquid chromatography on Superose 6 (Pharmacia) with 6 M guanidine HCl for elution. The gel filtration step removes non-GTF and other glucan-binding proteins from the GTF preparation, as evidenced by the fact that the protein bands observed after SDS-polyacrylamide gel electrophoresis were all associated with enzymatic activity after incubation of duplicate gels in sucrose. The *Streptococcus sobrinus* GTF preparation obtained after gel filtration on Superose 6 contained a mixture of GTF isozymes including GTF-I and GTF-S but was essentially free of other proteins. Approximately 90% of the glucan synthesized by this preparation was water-insoluble, under the conditions of the assay described below. This preparation was used for microparticle preparation, immunization experiments, enzyme assays and enzyme-linked immunosorbent assay (ELISA) measurement of antibody activity.

Microparticle preparation

S. sobrinus GTF was incorporated into the PLGA-based biodegradable carrier using 1% gelatin as a bioadhesive agent. Bioadhesives employing secondary forces are most relevant for temporary attachment to M-cell surfaces prior to endocytosis, taking advantage of electrostatic interactions including hydro-

gen bonding between charged hydrophilic groups (carboxyl, amino, sulfate), as well as hydrogen bonding involving uncharged hydroxyl groups (4). Mechanical bonding is facilitated by these secondary forces (12). Studies with mouse ligated loop models indicated that gelatin had superior bioadhesive properties compared with Eudragit and lectin; thus gelatin was selected for use.

The PLGA copolymer (Boehringer Ingelheim Chemicals, Mannheim, Germany) was characterized with respect to molecular weight distribution using gel permeation chromatography (Ultrastragel 100 Å and Ultrastragel Linear columns). A ratio of 75:25 lactide: glycolide was used in this experiment. A low density polymer foam was prepared by lyophilization of a polymer solution in glacial acetic acid. The polymer foam was cryogenically ground in a Tekmar Model A-10 analytical mill (20,000 rpm) equipped with a cryogenic well, cooled with liquid nitrogen, enabling low temperature particle size reduction.

S. sobrinus GTF (10%) and gelatin (1%) were dry mixed (w/w) with PLGA 75:25 polymer. The matrix was then compressed and extruded at a pressure of 15,000–20,000 psi and at a temperature of 45–55°C. High-pressure extrusions ensure that the protein is fully incorporated with the polymer lattice with a concomitant reduction in particle porosity: this minimizes premature release of the active agent. The sized PLGA/GTF/gelatin blend was loaded into a mold and extruded through a 2.4-mm-diameter die using a Compac Type MPC 40-1 hydraulic press. Following extrusion the matrix was again cryogenically ground in a Tekmar mill and sieved to retain particles less than 45 µm. Gelatin/PLGA microparticles that did not contain GTF were prepared under similar conditions for use as a control antigen preparation.

Immunization protocol

The mucosal immunogenicity of GTF/1% gelatin dry mixed with PLGA-75:25, compressed and Tekmar ground microparticles was measured in six groups (groups A through F) of Sprague-Dawley rats, using the protocol shown in Table 1. Prior to immunization, all rats were bled from the tail vein and salivated for 10 min by gravity collection (10 mg pilocarpine nitrate/kilogram rat weight) under ether anaesthesia. Immunization was initiated

Table 1. Immunization protocol

Groups	n	Route	Protocol			
			GTF (μ g)	Formulation	Primary immunization days	Secondary immunization days
A	6	intranasal	0	1% gelatin/PLGA	0, 7, 14, 21	103, 110, 117
B	6	intranasal	60	GTF/1% gelatin/PLGA	0, 7, 14, 21	103, 110, 117
C	5	intranasal	20	soluble GTF + alum	0, 7, 14, 21	103, 110, 117
D	6	intragastric	60	GTF/1% gelatin/PLGA	0, 7, 14, 21	103, 110, 117
E	4	subcutaneous	60	GTF/1% gelatin/PLGA	0	28
F	4	subcutaneous	5	soluble GTF + alum	0	28

when rats were approximately 45 days old. Four weekly primary mucosal immunizations and three weekly secondary mucosal immunizations were performed in groups A–D. Subcutaneously injected rats (groups E and F) were immunized in the vicinity of the salivary glands on days 0 and 28. Rats that were mucosally immunized with antigen in PLGA were given a three-fold higher dose than rats immunized intranasally with GTF in soluble form because the release data (Table 2) indicated that somewhat less than one third of the enzyme in the GTF-loaded microparticles was released *in vitro*. Animals injected with GTF/gelatin/PLGA were given the same GTF dose as those mucosally immunized with the gelatin-PLGA microparticles. All rats were bled and salivated on 1, 3, 7 and 11 weeks after primary immunization and 1, 3 and 7 weeks after secondary immunization. Serum IgG and salivary IgA antibody were then measured by ELISA.

Rats in groups A–C were intranasally immunized with 0.03 ml of microparticle mixture distributed equally between both nostrils with an Ependorf pipet. This dose was well tolerated by the intranasally immunized animals; thus, no anesthesia was required for antigen administration. Rats in group D were immunized intragastrically with 0.03 ml microparticle mixture via a 20 gauge, 1.5-inch intubation needle (Poo-

er & Sons, New Hyde Park, NY). Food was withdrawn 4–6 h before immunization and 0.1 ml of 0.2 M sodium bicarbonate was added immediately prior to antigen administration to reduce stomach acidity. Rats in groups E and F were subcutaneously injected with 60 μ g GTF/PLGA or 5 μ g GTF in aluminum phosphate.

ELISA

Serum IgG and salivary IgA antibodies were tested by enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (Flow Laboratories, San Francisco, CA) were coated with 0.2 ml of 0.5 μ g/ml of *S. sobrinus* GTF. Antibody activity was then measured by incubation with 1:400 and 1:10,000-fold dilutions of sera, or 1:4, 1:16 or 1:64-fold dilution of saliva. Plates were then developed for IgG antibody with rabbit anti-rat IgG, followed in sequence by alkaline phosphatase goat anti-rabbit IgG (Biosource, Camarillo, CA) and *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO). A mouse monoclonal reagent to rat α chain (Zymed, South San Francisco, CA) was used with biotinylated goat anti-mouse IgG (Zymed) and avidin-alkaline phosphatase (Cappel, West Chester, PA) to reveal levels of salivary IgA antibody to peptides. Reactivity was recorded as absorbance ($A_{405\text{ nm}}$) in a

micro plate reader (Biotek Instruments, Winooski, VT). Data are reported as ELISA units (EU) which were calculated relative to the reactivity of appropriate reference sera.

Measurement of *S. sobrinus* GTF enzyme activity

Nine milligrams of microparticles, initially loaded with 0.9 mg of GTF, were incubated at 37°C on a platform rocker in polypropylene tubes containing 1.6 ml of phosphate buffered saline, pH 6.5, with 0.2% bovine serum albumin and 0.2% sodium azide. At each experimental time point aliquots were removed, spun in a microcentrifuge, and the supernatants tested for released GTF. The release of GTF from microparticles was assayed by measuring the ability of the *S. sobrinus* GTF to catalyze the synthesis of glucan from sucrose. In the assay, 1.7 mg sucrose and 9 nCi of [14 C-glucose]-sucrose (approximately 17,000 cpm) are added to 0.1 ml of phosphate-buffered saline, pH 6.8 with added 0.02% sodium azide, 0.1% bovine serum albumin, and 0.3 mg dextran primer. Incubation proceeded for 5 h at 37°C, after which glucan was precipitated in 3 volumes of 95% ethanol and radioactivity determined as previously described (18). Assays were performed in duplicate.

Results

in vitro release of GTF from PLGA/1% gelatin microparticles

The release of GTF from the microparticles after incubation at 37°C in PBS-azide was measured by the ability of released GTF to incorporate 14 C glucose from labeled sucrose into glucan, as described in Material and methods. The activity of an amount of free GTF, equivalent to fully loaded microparticles and incubated under the same

Table 2. *In vitro* release of GTF from PLGA microparticles

Time of sample incubation at 37°C (h)	1% gelatin/PLGA (cpm 14 C-glucose) ^a	GTF/1% gelatin/PLGA (cpm 14 C-glucose) ^a	GTF activity released/total GTF activity added to microparticle
0.2		870	0.13
2	37	1740	0.26
20		1587	0.24
52	38	1226	0.18
96		1012	0.15

^a Release of GTF activity from microparticles was measured by the incorporation of 14 C glucose from 14 C-sucrose, as described in Material and methods.

conditions as the GTF PLGA microparticles, was also measured and used to determine the percentage of enzyme released. The results are shown in Table 2. The maximum release of active GTF from the microparticles was found to occur after 2 h of incubation under the conditions indicated. The amount released at this time corresponded to 26% of the GTF activity calculated to be incorporated into the equivalent amount of microparticles. This release rate was somewhat more rapid than that seen for microparticles containing a prototype α -amylase protein (39% release of enzyme activity at 19 h of incubation), but was considered to be desirable for mucosal immunization given the potentially limited period of exposure of the microparticles to the nasal or intestinal surfaces.

Salivary IgA antibody responses to GTF

The mucosal immune responses of animals immunized intranasally, intragastrically or subcutaneously with GTF or placebo in gelatin-coated microparticles or alum are shown in Fig. 1. Significant elevations in salivary IgA antibody levels to *S. sobrinus* GTF were observed only in the groups immunized intranasally on day 28. Five of six rats given the GTF/gelatin/PLGA microparticles intranasally had elevated salivary IgA antibody to GTF. Two of these animals had antibody levels which were detectable at salivary dilution of at least 1:128. Two of the five rats given GTF intranasally in alum had low but positive responses. However, the mean response of the alum group was less than 1/10 that of the PLGA groups at this time. The GTF/gelatin/PLGA intranasally administered group B antibody levels remained significantly elevated above the sham group A levels for at least 69 days of the primary response. Three months after initial intranasal immunization, salivary IgA antibody levels continued to be high in two of the six group B rats (74 ELISA units and 119 ELISA units), although the mean group B antibody level was not significantly greater than group A. In contrast to group B animals, the rats in groups E and F, who received a single subcutaneous injection of GTF in either microparticles or microparticles, did not show a salivary IgA antibody response until day 42, following a second injection on day 28.

Following a more limited secondary

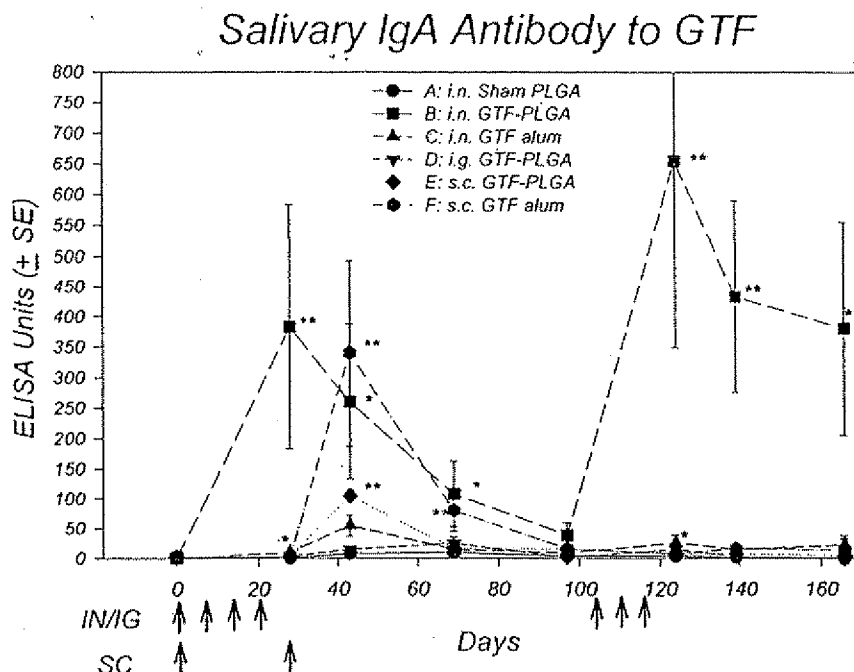


Fig. 1. Mean and standard deviations of salivary IgA antibody levels to *S. sobrinus* GTF for all groups throughout the full course of the experiment. Group designations and treatment are indicated in the legend. Intranasal/intragastric (IN/IG) or subcutaneous (SC) immunization events are indicated by arrows beneath the abscissa. The levels of statistical significance, compared with the sham-immunized group A are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; Kruskal-Wallis ANOVA on ranks).

mucosal exposure to antigen on days 103, 110 and 117 (Fig. 1), all rats intranasally immunized with GTF/gelatin/PLGA microparticles demonstrated a salivary IgA antibody response to GTF. Seven days after completion of the secondary immunization regime (day 124), the mean salivary IgA antibody response in this group was nearly twice that detected on day 28. Furthermore, mean salivary IgA antibody levels during the secondary phase of the response to intranasally administered GTF/gelatin/PLGA microparticles remained at or above peak primary antibody levels for at least 42 days. A low but significant increase in salivary IgA antibody was observed only on day 124 in the group C rats who had been given GTF in alum intranasally. No significant increase was observed in the intragastrically immunized group D. The salivary IgA antibody levels of groups E and F increased to significant levels after a second injection of GTF microparticles or GTF in alum on day 28. However, neither injected group achieved the level or duration of secretion of salivary IgA antibody seen after the second immunization regime given to the intranasally immunized group B.

Serum IgG antibody responses to GTF

Serum IgG antibody responses to GTF were also measured in all animals prior to immunization and throughout the course of the experiment (Fig. 2). Serum IgG antibody to GTF could be detected in all subcutaneously injected animals of either group E or F and in four of the six intranasally immunized rats of group B, 4 weeks after initial exposure to the antigen. No other group had significantly elevated serum IgG antibody to GTF at this time, although serum IgG antibody could be detected in at least two group C rats.

After a second injection of GTF in alum or microparticles, serum IgG antibody levels increased more than tenfold and remained high for the duration of the experiment (Fig. 2). The mean serum IgG response in the group B rats remained significantly higher than all other mucosally immunized groups throughout the primary immunization period and increased significantly from the peak primary level (day 28) after a second mucosal immunization regime. Serum IgG and salivary IgA antibody levels in the group B rats showed significant positive correlations (Spearman

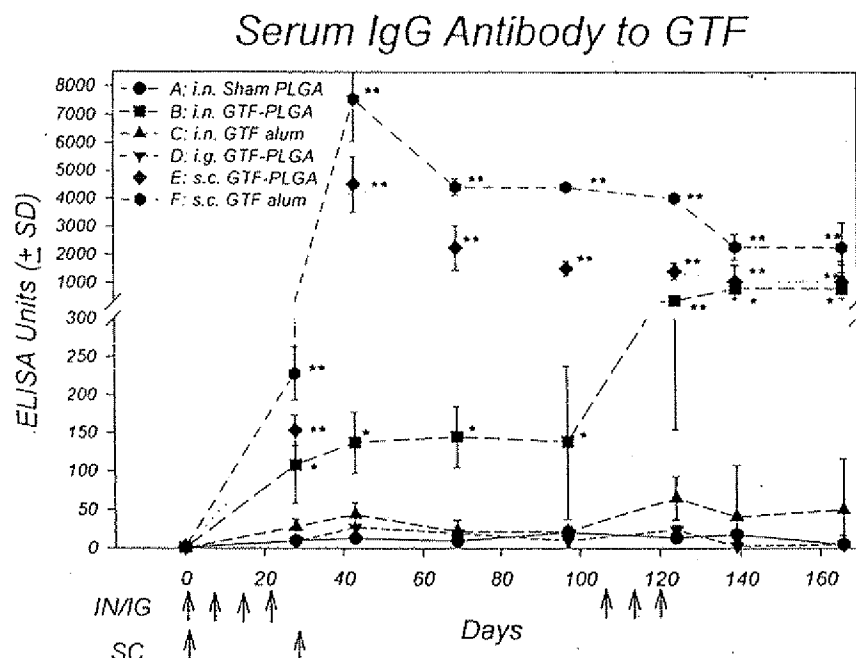


Fig. 2. Mean and standard deviations of serum IgG antibody levels to *S. sobrinus* GTF for all groups throughout the full course of the experiment. Group designations and treatments are indicated in the legend. Intranasal/intragastric (IN/IG) or subcutaneous (SC) immunization events are indicated by arrows beneath the abscissa. The levels of statistical significance, compared with the sham-immunized group A are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; Kruskal-Wallis ANOVA on ranks).

rank order correlation) after both primary ($r^2 = 0.996$) and secondary ($r^2 = 1.00$) immunization.

Discussion

The results reported herein support the hypothesis that the dry mix method of incorporation of *S. sobrinus* GTF into PLGA microparticles, followed by compression, extrusion and grinding do not radically affect the structure of this enzyme. This conclusion is based on the fact that significant enzymatic activity (26%) could be detected in the supernatant of GTF-PLGA microparticles incubated for 2 or more hours under the conditions described. This observation was supported by preliminary release experiments with α -amylase. Amylases are a group of enzymes that share with mutans streptococcal GTFs the ability to release glucose from polysaccharides. Recent modeling studies indicate that the catalytic domain of GTF bears striking homology with α -amylases with regard to secondary domain structure and with regard to the essential residues involved in catalytic activity (8). Thus α -amylase provided a useful prototype to measure the release

rate and effect of particle preparation on enzymatic activity. Up to 39% of the loaded α -amylase activity could be recovered after 19 h of incubation at 37°C in phosphate-buffered saline with azide (data not shown). The slightly higher amount of α -amylase activity compared with GTF activity released might be explained by the larger size of GTF. However, both sets of experiments indicate that the microparticle preparation method preserves much if not all of the native structure of these two proteins, based on the preservation of their enzyme activity. Thus, immunization with GTF microparticles could be expected to include immune responses to some conformationally dependent epitopes.

Based on the amount of release of enzymatically active GTF, intranasally immunized PLGA-GTF rats were given a three-fold greater dose (calculated from the amount of GTF initially added in the manufacturing process) than rats intranasally immunized with soluble GTF. It is conceivable that non-enzymatically active, but immunogenic GTF or fragments thereof could also be released, thereby increasing the actual dose. We have qualitative evidence from previous experiments that some GTF

protein remains in the microparticles for extended periods. Thus not all of the protein is released. Furthermore, if the GTF release data for the PLGA-GTF microparticles (26%) represents all the immunogenic GTF released, then the PLGA-GTF microparticle rats would have received only 78% of the dose received by the rats immunized intranasally with soluble GTF. These observations, taken together with the finding that the subcutaneously injected PLGA-GTF rats consistently demonstrated significantly lower salivary and serum responses than did the subcutaneously injected soluble GTF rats, despite the fact that they received a three-fold higher GTF dose (Table 1), supports the notion that the intranasally immunized PLGA-GTF rats did not receive a higher useful GTF dose than their soluble GTF immunized counterparts.

Intranasal administration of antigen has been shown to induce salivary IgA antibody (17) at levels that are generally superior to those induced intragastrically (21). In the present study, intranasal application of GTF-PLGA microparticles was far superior to other routes (intragastric or subcutaneous) or formulation (alum) for induction of salivary IgA immunity after primary or secondary immunization regimens. The superiority of the intranasal, versus the intragastric route for induction of salivary antibody may be a consequence of the relatively higher dilution of particulate antigen in the intestine or the greater potential for rapid breakdown of antigen in the gut versus nasal/tonsillar area. A degree of compartmentalization of the mucosal immune response may also account for this difference, in that nasal/tonsillar induction of mucosal immunity may favor a greater expression of IgA antibody in the saliva than would induction of gut-associated lymphatic tissue, which favors a predominantly intestinal expression of antibody activity (9).

Microparticle bioadhesiveness may increase the dose of antigen available to induce local immune responses by increasing the time that antigen-laden biodegradable particles are in contact with mucosal surfaces. Montgomery & Rafferty (10) have shown that oral administration of bioadhesive degradable starch microparticles containing dinitrophenyl-bovine serum albumen, in combination with L- α -lysophosphatidylcholine as a penetration enhancer, po-

tentiated long-lived salivary IgA responses, compared with antigen delivered in soluble form. In the present study both GTF-loaded and control PLGA preparations given intranasally or intragastrically contained gelatin to increase the bioadhesive properties of the microparticles. Although the present experiments do not clarify the extent to which the use of gelatin improved the immune response, previous work with the mouse ligated intestinal loop model (3) indicated that incorporation of gelatin enhanced microparticle uptake into phagocytic cells within the follicle associated epithelium.

Intranasally administered GTF-PLGA microparticles induced a salivary IgA antibody response to GTF that was relatively long lasting compared with other routes and modes, especially after secondary stimulation (Fig. 1). This mucosal IgA antibody response had aspects of anamnestic in that the peak secondary response was higher than the peak primary response (604 ELISA units versus 383 ELISA units). Furthermore, salivary IgA antibody was detected at a higher level for a longer period of time in the secondary, compared with the primary, phase of the response to the intranasally applied GTF-PLGA microparticles. Another important feature was that the secondary exposure to the intranasal GTF-PLGA microparticles converted all the responses of rats in whom either no or low primary salivary IgA antibody responses had been detected. This suggests that repeated intranasal application of appropriate antigen in these bioadhesive microparticles could be expected to induce an immune response in most subjects, despite the typically wide range in secretory immune responses seen to indigenous and artificial antigens.

A significant serum IgG antibody response was also seen in several rats that were intranasally immunized with GTF-PLGA microparticles (Fig. 2). This response was highly associated with the degree of salivary IgA response among animals of this group. The serum IgG responses seen in the most responsive group B rats were within the range of those seen in the systemically immunized rats by the end of the experiment (day 169; Fig. 2). The precise mechanisms for these responses are as yet unclear. One explanation would be that in the most responsive group B rats, some GTF-PLGA microparticle

fragments were phagocytized and carried to the cervical lymph nodes, where they continued to supply antigen-presenting cells with GTF for induction of systemic immunity. In this regard, Wu & Russell (21) have reported that antibody-secreting cells appeared in both the superficial and posterior cervical lymph nodes shortly after completion of intranasal administration of soluble *Streptococcus mutans* Ag I/II conjugated to cholera toxin B subunit.

The enhancement of the expression of immunity within the oral cavity after delivery of GTF in bioadhesive microparticles indicates that this immunization regime may be particularly efficient in the induction of antibody that could interfere with the colonization and accumulation of cariogenic mucosal streptococci in the oral cavity. Protection could be expected not only from the enhancement of salivary immunity but also from systemic IgG antibody that enters the oral cavity via the gingival crevicular fluid route. Intranasal application of similar formulations of bioadhesive microparticles, loaded with appropriate antigen, also holds promise for the enhancement of immunity to a variety of upper respiratory and oral infections.

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